

Microbiology of acute arthropathies among children in Argentina: II. *Chlamydia trachomatis* and *pneumoniae*

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Abstract

No analysis has been carried out on the impact of *Chlamydia* in pediatric patients with arthritis. The present study was conducted to investigate whether *Chlamydia trachomatis* and *Chlamydia pneumoniae* could be identified as a triggering infection in 33 pediatric patients including adolescents with acute joint disorders by using an extensive laboratory approach. Synovial fluid, nasopharyngeal secretion, and urethral swab samples were screened for the presence of *Chlamydia* by culture, enzyme immunoanalysis and immunofluorescence assay. In addition, in synovial fluid samples, detection of *Chlamydia trachomatis* and *pneumoniae* DNA was performed by nested-PCR. Specie-specific antibodies were tested in sera by microimmunofluorescence method. Of the 33 patients, 3 (9%) had joint disorders associated with *Chlamydia*. In synovial fluid samples of 2 (6%) patients with evidence of reactive arthritis we found *C. trachomatis* antigens and specific nucleic acids. The presence of this organism and its antigenic components was also confirmed in urethral swabs from these patients. *C. pneumoniae* DNA and antigens were found in joint fluid from one patient (3%) who presented inflammatory arthritis and respiratory infection. Bacterial isolation and antigenic detection gave positive result in nasopharyngeal secretion from this patient. Antibody titers against *C. trachomatis* or *C. pneumoniae* were observed in paired sera from these 3 patients showing evidence of preceding infection. We conclude that the diagnosis of *Chlamydia*-associated arthritis was based on the clinical picture, finding of the bacteria, nucleic acids and/or its antigens at the site of inflammation and relevant exclusion of other triggering microbes. Our study represents the first data from Argentina linking Chlamydial infection to joint disorders in children and adolescents and may contribute to early diagnosis to prevent the evolution to more advanced clinical forms.

Keywords: Arthropathies. *Chlamydia trachomatis*. *Chlamydia pneumoniae*. Children.

INTRODUCTION

Recent molecular genetic studies have elucidated several aspects of the biology of *Chlamydia trachomatis* and *Chlamydia pneumoniae* infection in the synovial context, and these new

data provide explanations for some characteristics of the arthritis associated to these organisms.^{1, 2, 3, 4}

Urogenital infection with *C. trachomatis* has long been associated with development of an inflammatory joint disease designated as reactive arthritis. Indeed, reactive arthritis can

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be also triggered by prior gastrointestinal infection with various species of genera *Salmonella*, *Yersinia*, *Campylobacter* and others,^{5, 6} suggesting that the primary focus of infection be through the mucosal membrane in the gut or in the urogenital tract. Originally, diagnosis of *Chlamydia*-associated reactive arthritis was based on the culture of the organism from urogenital samples. However, due to the high prevalence of urogenital chlamydial infections, demonstration of *C. trachomatis* in the urogenital tract cannot prove the chlamydial etiology of the arthritis.^{7,8,9,10} Furthermore, as screening methods for identification of *Chlamydia* have improved, patients who have chlamydial antigens or nucleic acids in the joint, but do not meet American College of Rheumatology (ACR)¹¹ criteria for reactive arthritis, are being recognized as simply having *Chlamydia*-associated arthritis. Many of those patients do have oligoarthritis and probably have similar disease mechanisms.^{12, 13, 14}

Direct approaches to identification of causative organisms have centered on evidence of the bacterium in the joint. Immunofluorescence studies have identified persistent microbial antigens in patients with reactive arthritis subsequent to *Chlamydia*-related joint disease.¹ In addition, molecular biologic techniques such as polymerase chain reaction (PCR) have been increasingly used to identify a potential bacterial etiology in arthritis.

According to recent reports^{1,3} *C. pneumoniae* is known to cause a strong inflammatory response at site of its residence. The presence of this pathogen may also have a role in the etiology of reactive arthritis.³ No analysis has been carried out on the impact of this organism in pediatric patients with acute arthritis.

In Argentina, there are not statistical data of the chlamydial infection prevalence in joint disorders, especially in children.

Here, we report our first findings of *C. trachomatis* and *C. pneumoniae* from synovial fluid samples of pediatric and adolescent patients, its serum antibodies titers and clinical characteristics. Moreover, we discuss the contribution of our results to early diagnosis of this infection.

PATIENTS AND METHODS

Selection of children

Criteria for inclusion in this study were the following. In patients attended at the Ricardo Gutierrez Children's Hospital who had acute arthropathies and/or inflammatory low back pain. During a one-year period, 45 samples of synovial fluid, blood, urethral swabs and nasopharyngeal secretion were collected from 33 hospitalized patients. Of these 33 patients, 14 were males and 19 females ranging in age from 1-17 years in males, and from 1-13 years in females. All patients were asked about recent respiratory, enteric or urogenital infection within a month before the onset of arthritis.

Presentations included: acute suppurative arthritis (n=21), juvenile rheumatoid arthritis (n=3), reactive arthritis (n=3), osteomyelitis caused by metastatic bone tumor (neuroblastoma) (n=2), post-traumatic osteomyelitis (n=2), and congenital arthrogryposis (n=1), undifferentiated oligoarthritis (n=1), according to ACR criteria.

All included cases were subjected to a comprehensive study involving microbiologic, immunologic and clinical examinations. Radiologic and echographic studies were performed in all patients, and biopsy, arthrography and/or magnetic resonance imaging of the joint were done in some cases.

Patients with arthritis associated to *Chlamydia* infection were treated with nonsteroidal anti-inflammatory drug and clarithromycin (250-500 mg p.o.twice day).

Sample preparation

Joint fluid, nasopharyngeal secretion and urethral swab samples were collected in sterile containers which carried 1ml of culture transport medium and sent to the laboratory to detect *Chlamydia* by the following methods:

1. LPS-direct enzyme immunoassay (EIA): which detects Chlamydial lipopolysaccharide

(LPS) shared by all members of the genus (*Chlamyfast*®^{15, 16} test International Microbio, Signes, France).

2. Direct fluorescence-antibody (DFA) technique: specimens were tested by DFA using a labeled monoclonal antibodies directed against *C. trachomatis* Major Outer Membrane Protein (MOMP) (Microtrack Syva Co, USA) to confirm EIA results.

3. Indirect fluorescence-antibody (IFA) technique: synovial fluid specimens were tested using monoclonal antibodies directed against *C. pneumoniae* Major Outer Membrane Protein (MOMP). A suitable secondary conjugate was used for indirect immunofluorescence staining (DAKO *C. pneumoniae* anti-bacterium research reagent and DAKO Rabbit anti-mouse immunoglobulin-FITC, DAKO Co, USA).

4. Isolation in cell culture: Inoculation of clinical specimens into Cycloheximide treated Mc Coy and HL cells to isolate *C. trachomatis* and *C. pneumoniae* respectively were performed. Then, inoculum centrifugation onto the cell monolayer, incubation for 48-72 hs and staining using a labeled monoclonal antibodies against LPS of the Chlamydial particle was done (Imagen *Chlamydia* - DAKO Co, USA).

C. trachomatis and *pneumoniae* control strains were provided by the Carlos Malbrán National Microbiology Institute.

5. Nested-PCR: synovial fluid samples of all enrolled patients were screened using polymerase chain reaction assay. Briefly, the synovial fluid pellet was thawed, resuspended in 0.1M NaCl, 1mM EDTA, 10mM Tris-HCL (pH8.0), 0.5% Tween 20, and digested overnight at 56°C using 1 mg proteinase K per ml (Sigma, Munich, Germany). The DNA extraction was performed as described elsewhere¹⁷ by using cetyltrimethylammonium bromide and QIAEX specimen preparation kit (Quiagen, Hilden, Germany) and the extracted DNA was resuspended in 60ul distilled water. Twenty microliters was used for outer PCR while 10 microliters of the reaction product was

used for nested PCR. PCR extraction and amplification were performed in separate rooms. Each PCR included 2 negative controls and 2 positive controls. The 2 negative controls consist of 1 rheumatoid arthritis fluid sample also passing through the DNA extraction procedure and 1 buffer sample without DNA. As a positive control a rheumatoid arthritis synovial fluid sample spiked with either ~ 100 *C. trachomatis* or *C. pneumoniae* elementary bodies per ml was used. Visualization of the amplification product was performed by 2% agarose gel electrophoresis and ethidium bromide staining under ultraviolet light. A sample was considered positive if there was a visible amplification product of correct length in 2 independent PCR test, with correct positive and negative controls.

C. trachomatis nested PCR targeting the major outer membrane protein consisted of 40 cycles (Denaturation: 90 seconds at 94°C, Annealing: 120 seconds at 55°C, Extension: 120 seconds at 72°C) for an outer PCR product and 40 cycles (Denaturation: 90 seconds at 94°C, Annealing: 120 seconds at 60°C, Extension: 120 seconds at 72°C) for a 152 base pair (bp) nested product.

Detection of *C. pneumoniae* DNA was performed by a nested PCR targeting the *ompA* gene¹⁸ consisting of 40 amplification cycles for a 333 bp product and 30 cycles for a 207 bp nested product. The 207 bp product was confirmed as *C. pneumoniae* by hybridization with a specific fluorescein-labeled oligonucleotide probe (ECL, Amersham).¹⁸

Serology

Sera samples were collected at entry and stored at -20°C. For serological confirmation, IgM and IgG antibodies against *C. trachomatis* were determined in serum samples by a modification of microimmunofluorescence method (MIF) described by Wang and Grayston.¹⁹ *C. pneumoniae*-specific antibodies were measured by MIF test in slides containing purified elementary bodies as antigen.²⁰

All sera were tested in serial dilutions from 1:32 for IgG antibody, and screened for IgM antibodies in 1:16 dilution with fluorescein isothiocyanate-conjugated anti-human immunoglobulins.

All initially IgM positive serum samples were absorbed with IgG inactivation reagent (IgG inactivation reagent, Orgenium Laboratories, Helsinki- Finland) and retested in MIF assay at serial dilutions. Antibodies titers of = 1:32 in the igM fraction or = 1:2000 in the igG fraction or an equal or greater than 2 fold titer change in antibody level were considered significant evidence of exposure to *C. trachomatis*.

Antibodies titers of = 1:16 in the igM fraction or = 1:512 in the igG fraction or an equal or greater than 2 fold titer change in antibody level were considered of ongoing *C. pneumoniae* infection.

Other assays

Blood samples were subjected to a hematological examination (hemoglobin concentration, red and white blood cell count, differential leukocyte count, erythrocyte sedimentation rate). Concentration of complement factors C3 and C4, immunoglobulin titers, rheumatoid factor, HLA-B27 and antinuclear antibodies were determined in serum samples.

In synovial fluid and urethral swab samples chemical and standard bacteriological culture were performed. In nasopharyngeal secretions, the presence of *Adenovirus*, *Respiratory syncycial virus*, *Influenza A* and *Human Parainfluenza virus* and *Mycoplasma pneumoniae* were also tested.

For objective interpretation, the evaluation of the results was made in a blinded manner.

RESULTS

Of the 33 patients 12 (36%) had joint disorders associated with pathogens present at the site of inflammation (TABLE 1). *Mycoplasma* and diverse bacteria identified in synovial fluid samples were described elsewhere.²¹

Organisms	N° of patients	Isolation site
<i>Ureaplasma Urealyticum</i>	1	Synovial fluid
<i>Mycoplasma hominis</i>	2	Synovial fluid
<i>Mycoplasma pneumoniae</i>	1	NPS
<i>Mycoplasma hominis</i> + <i>Staphylococcus aureus</i>	1	Synovial fluid
<i>Chlamydia trachomatis</i>	1	Synovial fluid** Urethra
<i>Chlamydia pneumoniae</i>	1	Synovial fluid NPS*
<i>B hemolytic Streptococcus group A</i>	2	Synovial fluid
<i>Staphylococcus aureus</i>	1	Synovial fluid
<i>Staphylococcus coagulase negative</i>	1	Synovial fluid
NONE	21	Synovial fluid

TABLE 1 – Organisms isolated from 33 pediatric patients with joint disorders

* NSP: nasopharyngeal secretion

** Antigen and DNA detection by enzyme immunoanalysis/ direct fluorescence assay and PCR respectively

C. trachomatis antigens and DNA were detected in synovial fluid samples of 2 (6%) patients. In addition, *C. pneumoniae* antigens and DNA were found in joint fluid from one patient (3%). *C. trachomatis* and *pneumoniae* DNA of all PCR-positive samples gave strong reproducible amplification signals (FIGURE 1). Detection of *Chlamydia* antigen in joint fluid by EIA commercial kit were confirmed by DFA and IFA, however, *Chlamydia* culture was negative in such clinical samples (TABLE 2). *C. trachomatis* was detected in urethral swab specimens in 2 patients by cell culture, EIA and DFA. (TABLE 2) These cases presented polyarthrititis affecting hip, knees and wrists and urinary tract symptoms 3 to 4 weeks preceding arthralgia. None of these patients had previous respiratory infection.

Antibody titers considered positive for active *C. trachomatis* infection were observed in paired sera which were taken at 25 days and 2 months after onset of arthralgias. One of them had a genetic predisposition for arthritis with a positive blood test for HLA-B27 and presented extra-articular features. Thus, in these patients a typical clinical picture for reactive arthritis was diagnosed. (TABLE 2)

C. pneumoniae culture, EIA and IFA gave positive result in nasopharyngeal secretion from a 6 year old child who developed undifferentiated oligoarthritis affecting ankles and knees. This patient presented a lower respiratory

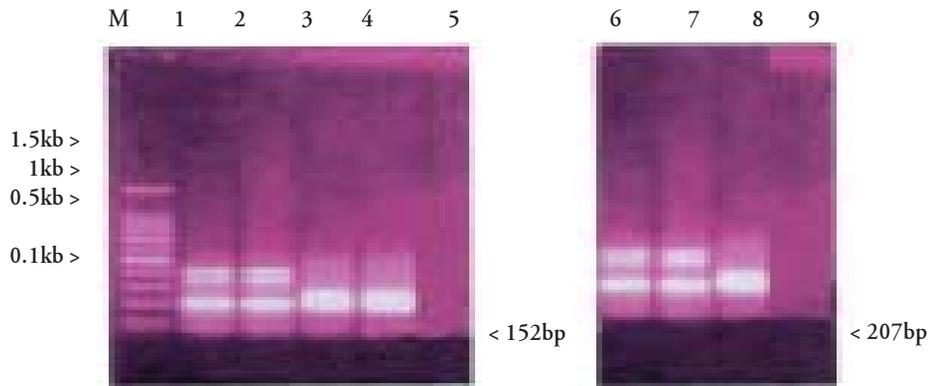


FIGURE 1 – Visualization of polymerase chain reaction (PCR) products by 2% agarose gel electrophoresis and ethidium bromide staining under ultraviolet light

M, DNA marker VIII (Boehringer, Ingelheim, Germany);
lanes 1 and 2, Positive controls (*C. trachomatis* in synovial fluid);
lanes 3 and 4 Clinical specimens containing *C. trachomatis* DNA;
lane 5 Negative control (synovial fluid);
lanes 6 and 7 Positive controls (*C. pneumoniae* in synovial fluid);
lane 8 Clinical specimen containing *C. pneumoniae* DNA.
lane 9 Negative control (Buffer sample without DNA).

Case	Sex/ Age (yr)	Diagnosis	Preceding Symptoms	Organism	Site or Specimen tested	<i>Chlamydia</i> detection method				<i>Chlamydia</i> Serology				Adnormal Findings
						CC	EIA	IFA	PCR	IgM Day 25	IgC Day 60	IgM Day 60 aprox	IgG	
1	M/13	Reactive arthritis	Urethritis	<i>Chlamydia trachomatis</i>	Urethra Knee Hip Wrist	+	+	+	ND	1:32	1:64	<1:32	1:128	Leukocytosis High ESR Abnormal Urianalysis Elevated Ig Level
2	M/17	Reactive arthritis	Urethritis Extra-articular Features	<i>Chlamydia trachomatis</i>	Urethra Knee Wrist	+	+	+	ND	<1:32	1:128	<1:32	1:512	Leukocytosis High ESR Abnormal Urianalysis Elevated Ig Level + HLA-B27
3	F/6	Undifferentiated oligoarthritis	Lower respiratory Infection Extra-articular features Low back pain	<i>Chlamydia pneumoniae</i>	NPS Knee Ankle Urethra	+	+	+	ND	1:32	1:64	<1:32	1:256	Leukopenia High ESR Elevated ig Level

TABLE 2 – Clinical data on 3 patients with *Chlamydia* associated arthritis

CC: Convencional culture
ELA: enzyme immunoanalysis
DFA: Direct fluorescence assay
ND: not done
ESR: Erythrocyte sedimentation rate Ig: immunoglobulin

infection 2-4 weeks before the onset of synovitis, extra-articular features and low back pain. Moreover, titers of IgM and IgG *C. pneumoniae* antibodies showed evidence of preceding infection. She did not have microbiological/serological findings for conventional infections associated with reactive arthritis (*Salmonella*,

Yersinia, *Campylobacter*) and had no urethritis or enteritis prior to arthralgias. (TABLE 2)

Adenovirus, *Respiratory syncicial virus*, *Influenza A* and *Human Parainfluenza virus* were not detected in nasopharyngeal secretion from these 33 patients. The most frequent symptoms related to *Chlamydia* associated arthritis were

fever, motor weakness and marked limitation of joint movement. Complication such as carditis, iritis and conjunctivitis were not observed. Laboratory findings such as high erythrocyte sedimentation rate and elevated immunoglobulin levels were shared by all of three patients. Hypercomplementemia, Latex-rheumatoid arthritis, anti-streptolysin test, and anti-nuclear antibodies were not observed. We could note that these studied patients were in disease remission at follow-up.

DISCUSSION

Previous studies have demonstrated the presence of a wide variety of bacterial species in the synovia of arthritis patients,^{22, 23, 24, 25} raising the question of whether organisms identified might have some association with the patients' various joint diseases. Detection of the microbe or its components at the site of primary infection or at the joint would be optimal to confirm the infectious etiology of arthritis.

In this study *C. trachomatis* and *C. pneumoniae* antigens and DNA were detected from joint fluid of 2 and 1 of 33 patients evaluated, respectively. *C. trachomatis* was isolated from urethral swabs in 2 adolescents who showed urethritis before the onset of arthralgia. In addition, *C. trachomatis* specific IgM and IgG antibodies were also documented in paired sera from these patients. A higher prevalence of *Chlamydia* associated arthritis was found in adolescents from 13 to 17 years old, probably due to sexually acquired infection. However, due to the high prevalence of urogenital chlamydial infections, demonstration of *C. trachomatis* in the urinary tract cannot solely prove the chlamydial etiology of arthritis.²⁶ For this reason, we were encouraged to investigate the presence of *Chlamydia* in the site of inflammation.

Synovial fluid from patients with either acute or chronic *Chlamydia*-associated arthritis are usually culture negative. This standard laboratory test for the organism probably fails because, in its synovial context, *Chlamydia* might be in a persistent state, rather than a state of

normal vegetative growth.^{1, 27} During persistent infection, genes whose products are required for chlamydial DNA replication are expressed, but transcription of genes whose products are required for cytokinesis are severely downregulated.²⁸ Indeed, enzyme immunoanalysis and immunofluorescence assays have identified Chlamydial antigen in synovial fluid from these patients with joint disease subsequent to *Chlamydia*. As the performance profiles of the commercially available EIAs vary considerably and have been validated for use with cervico-urethral samples, an approach to the result confirmation was to test the specimens by a second test based on a different principle, in this case, an immunofluorescence assay based on MOMP specie specific detection to confirm an LPS-based EIA. Since EIA detects the genus specific LPS which is known to crossreact with bacterial and other antigens, the validity of our data is emphasized by PCR results. No DNA was detected in synovial fluid samples of patients with definite rheumatoid arthritis (control negative), and intense precautions were taken to avoid laboratory contamination.

The presence of Chlamydial DNA in joint fluid of these reported patients raises the question of whether this proves bacterial etiology of the arthritis and if this is indicative of a persistent infection. Additional evidence supporting a bacterial etiology, such as specific cytokine patterns or bacteria-specific-T cell responses, would be helpful. The viability of organisms detected in the joint could additionally be tested by detection of short-lived RNA transcripts using reverse transcriptase PCR.

With regard to *C. pneumoniae*, this organism was found as respiratory pathogen in nasopharyngeal secretion from one child with lower respiratory symptoms preceding arthritis and serological evidence of recent infection.

In particular *C. pneumoniae* detection as respiratory pathogen at the primary focus of infection, the mucosal membrane, would be optimal to suggest the initiation or exacerbation of joint disease in the six year old patient. Although it is well documented that *C. pneumoniae* can induce a powerful inflammatory

response in sites of its residence,²⁹ no direct evidence available to date unequivocally links synovial inflammation with presence of *C. pneumoniae*.² In this regard, an infection-related origin is strongly suggested by the presence of *Chlamydial* antigens and DNA in the joint fluid of the patient with undifferentiated arthritis who developed high antibody titers against *C. pneumoniae*. Therefore, we suggest that *C. pneumoniae* should be included among the triggering factors in the etiology of reactive arthritides.

Antibodies to *C. trachomatis* and *C. pneumoniae* were measured by specific assay, the MIF test.¹⁹ This assay remains, so far, the only sensitive and specific serological test for Chlamydial infections and cross-reactions because the antibodies against other microbes are uncommon. The specificity of the MIF test is also observed in the present study. None of the patients with *C. trachomatis* infection had serological evidence

for *C. pneumoniae* infection and viceversa. Besides the established serological criteria of *Chlamydia* infection, we conclude that the diagnosis of *Chlamydia*-associated arthritis was based on the clinical picture, finding of bacteria, nucleic acids and/or its antigens in the site of the inflammation and on relevant exclusion of other triggering microbes.

Our study represents the first data from Argentina linking Chlamydial infection to joint disorders in children and adolescents. Although we attempted to develop a wider clinical and laboratory criteria for the differential diagnosis of *Chlamydia*-induced joint disorders in pediatric patients, further data involving a greater number of cases are needed to achieve this goal. Nevertheless, the results of the present work may contribute to early diagnosis and initiation of specific treatment to prevent the evolution to more advanced clinical forms.

Microbiologia de artropatias agudas em crianças na Argentina: II. *Chlamydia trachomatis and pneumoniae*

Resumo

Não se tem conhecimento da realização de análises sobre o impacto da Chlamydia em pacientes pediátricos com artrite. O presente estudo teve como objetivo investigar se a Chlamydia trachomatis e a Chlamydia pneumoniae poderiam ser identificadas como causadoras de infecções em 33 pacientes pediátricos, inclusive adolescentes, com doenças agudas da articulação, através de exames laboratoriais extensivos. As amostras de líquido sinovial, de secreção nasofaríngea e swab uretral foram rastreadas para verificar a presença de Chlamydia, através de testes de cultura, imunoanálises enzimáticas e imunofluorescência. Além disso, nas amostras de fluido sinovial, aplicou-se o nested-PCR para a análise do DNA de Chlamydia trachomatis e C. pneumoniae. Os anticorpos espécie-específicos foram testados nos soros através do método de microimunofluorescência. Dos 33 pacientes, 3 (9%) apresentaram problemas agudos nas articulações associados à Chlamydia. Nas amostras de fluido sinovial de 2 (6%) pacientes com evidência de artrite reativa, foram encontrados antígenos de C. trachomatis e ácidos nucléicos específicos. A presença desses organismos e de seus componentes antigênicos foi confirmada ainda nos swabs uretrais dos pacientes. O C. pneumoniae DNA e os antígenos foram encontrados no fluido da articulação de um paciente (3%) que apresentava artrite inflamatória e infecção respiratória. O isolamento bacteriológico e a constatação antigênica apresentaram resultados positivos na secreção nasofaríngea do paciente. Foram observados títulos de anticorpos contra C. trachomatis e C. pneumoniae nas duas amostras dos três pacientes que apresentaram infecções anteriores. Concluímos que o diagnóstico de artrite associado à Chlamydia foi baseado no quadro clínico, na descoberta de bactérias, de ácidos nucléicos e/ou de seus antígenos no local da inflamação, assim como pela exclusão relevante de outros micróbios. Nosso estudo representa a primeira coleta de dados na Argentina, em que se vincula a infecção por Chlamydia a problemas de articulações em crianças e adolescentes. Além do mais, ele pode contribuir para o diagnóstico precoce na prevenção da evolução de formas clínicas mais avançadas.

Palavras-chave: Artropatias. Chlamydia trachomatis. Chlamydia pneumoniae. Crianças.

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